

HABITUATION IN *LIMULUS* ABDOMINAL GANGLIA¹

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The phenomenon of habituation, or response decrement with repetitive stimulation, is generally considered to be a form of learning and distinguishable from response fatigue and receptor adaptation (Bullock and Quarton, 1966). It may be argued that it is equally important for an animal to be capable of "learning not to respond" (Thorpe, 1966, page 60) as it is for the animal to acquire new responses or potentiate existing ones. Habituation may be a form of learning common to all organisms—its demonstration in *Phycomyces* (Ortega and Gamow, 1970), protozoa (Appelwhite, Gardner and Lapan, 1969; Wood, 1970), coelenterates (Rushforth, 1967), planarians (Westerman, 1963), several arthropods (for examples and reviews, see McConnell, 1966; Thorpe, 1966; Thorpe and Davenport, 1965; Treherne and Beament, 1965) as well as in the vertebrates (Sharpless and Jasper, 1965; Thompson and Spencer, 1966) attests to a phylogenetic ubiquity.

In *Limulus polyphemus*, previous research has suggested that this "living fossil" also possesses the capacity for habituation of visual reflexes (Corning and Von Burg, 1968). Stimulation of a lateral eye elicits leg movements that are mainly contralateral. With repeated stimulation, the frequency of reflex activation was found to diminish. Evidence for a central locus of the response decrement was obtained when tests of the unstimulated eye demonstrated an initially lower probability of response elicitation in the opposite set of legs. Attempts to demonstrate other types of learning in *Limulus* have been negative or not overly convincing (Makous, 1969; Smith and Baker, 1960; Wasserman and Patten, 1969).

In the present investigations, we have attempted to obtain electrophysiological evidence of habituation in *Limulus* by studying the effects of repetitive tactile stimulation on the activity of abdominal ganglia efferents. The abdominal ganglia of *Limulus* provide an experimentally amenable system for studying habituation and its underlying mechanisms (Von Burg and Corning, 1969; 1970). The entire ventral cord region can be exposed in 15-20 minutes, and the preparation is viable for at least 6 hours although in some cases it has been electrophysiologically active for 24-48 hours. The first four abdominal ganglia are large and anatomically differentiated, permitting lesions and electrode placements to be made at readily identifiable sites.

Each ganglion has two pairs of nerves: dorsal and ventral. The ventral nerves carry efferents that control gill movement (Hyde, 1893), and afferents from the surfaces of the gill books and carapace (Patten and Redenbaugh, 1900). The dorsal nerves contain cardioinhibitors and cardioaccelerators as well as sensory fibers from the lateral spines (Corning, Von Burg and Lahue, 1970; Von Burg and Corning, 1970). Tactile stimulation of the gill books will produce a marked increase in dorsal nerve output and a transient inhibition in heart rate. In the intact system, tactile stimulation will affect the activity of dorsal nerves in the other abdominal ganglia as well as the output of the ipsilateral and contralateral

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dorsal nerves in the same segment. However, the sensory information carried by afferents in each ventral nerve is derived solely from that segment and on the side that is stimulated. Thus, sensory information is segmental in origin whereas the consequences of this activation appear to be diffused throughout the central ganglia.

The direct examination of habituation in the central nervous system permits a more precise definition of afferent and efferent bioelectrical activity during stimulation. Presumably, if habituation is a central phenomenon, repetitive stimulation should produce response decrements in the ganglion outputs and have little effect on the activity of the sensory side. In addition, by studying the arthropod central nervous system with its segmented ganglionic masses, it is possible to examine the habituation process in the single ganglion, groups of ganglia, and in the intact system. These types of analyses can provide information concerning the relationship between neurological complexity and the acquisition and retention characteristics of the habituation process.

METHODS

Adult male and female specimens of *Limulus polyphemus* were procured from the Marine Biological Laboratory, Woods Hole, Massachusetts, April through December. The animals ranged from 6–8 inches in width as measured across the widest part of the cephalothorax. They were held in tanks of artificial salt water ("Instant Ocean") at a specific gravity of 1.022, pH of 7.8–8.3, and temperature of 15 to 18° C. The water was constantly filtered and for 20 min every hour there was heavy aeration.

For experimentation each subject was affixed, dorsal side down, to a board. This was most easily accomplished by driving nails through the peripheral edges of the carapace into the board. All walking legs were removed at the coxal-trochanter joint. This is necessary to reduce movement artifact during recording, and to prevent the legs from interfering with the electrodes or stimulating apparatus. The gill books were then split medially and the ventral surface of the opisthosoma opened along the midline from the last gill book forward past the operculum. The abdominal endochondrites were sectioned and, after lateral displacement of muscle and connective tissue, the ventral cord was completely exposed.

All recordings were made from the dorsal and ventral roots of the first four abdominal ganglia. Measures of ganglion output were made in dorsal nerve efferents. These are very responsive to tactile stimulation of the hair bristles along the ventral surface of the animal and the gill book surfaces. To obtain reliable recordings of the activity in these roots it was necessary to remove the membrane around the nerve. An incision was made with an eye scalpel and the membrane peeled away with fine dissecting forceps. A small portion of the root was nipped and the proximal end drawn into a suction electrode with an opening of approximately 10–15 μ . Tactile information enters the abdominal ganglia via the ventral roots. When recording the degree of afferent activity elicited by stimulation, the ventral nerve was dissected free to a point just before it entered the gill book. At this point the nerve was severed and the distal end of the bundle was drawn into a suction electrode.

Stimulation was provided by a light puff of air directed upon the ventral surface of the gill books through a glass pipette with a 1 mm tip diameter. The pipette tip was usually 5–7 mm from the gill book surface. Air pressure was provided by an air pump and the duration of the air flow was controlled by an Asco 2-way air valve activated by a square wave stimulator. The air puff was 0.5 sec in duration and was delivered once every 0.73 sec. Each animal received 3 blocks of stimulation with each block consisting of 360 puffs. The first block is designated as an "acquisition" phase. Three minutes after the termination of the first block, a second series of 360 puffs was delivered; this block is referred to as the "3-min retention test." Nine minutes after the completion of the second block the third block, or "9-min retention test," was initiated. At the completion of the last air puff for each block the stimulated point was given one of three "dishabituation" stimuli: a drop of water, a stronger puff of air, or a touch with a blunt dissecting probe. These stimuli provided tests for fatigue, *i.e.*, if response decrements represent true habituation, then stimuli of different intensity or quality should still be effective.

Four major preparations were studied. One group (14 animals) was habituated with a totally intact central nervous system. The stimulating and dorsal nerve recordings were accomplished at the same segment as well as different segments. However, no more than two ganglia intervened between the afferent input and the dorsal nerve that was recorded since the experiments were limited to the first four abdominal ganglia. A second group (15 animals) was habituated with the ventral cord region isolated from the rest of the central nervous system. As in the intact group, stimulation and recording were carried out at the same segment as well as over different segments. In a third group (14 animals), habituation was attempted in a ganglion that had been completely isolated from the central nervous system. In this group, the stimulation and recording were necessarily restricted to the same neuromere. To determine whether a response decrement in a ganglion output could be due to sensory adaptation or fatigue, recordings were made in sensory branches of the ventral nerve that innervated the gill book surface. In these preparations (4 animals), the mode and frequency of stimulus delivery were the same except that the recordings were made in the afferent fibers.

The bioelectrical activity was amplified with a Grass P511 ac amplifier with a HIP-511C high-impedance probe and stored on magnetic tape for later analysis. Unit activity was counted with a Ferch variable voltage gate and Hewlett-Packard high-speed counter. A Honeywell Model 906C Visicorder recorded both the raw data and the gate output simultaneously for a visual record of the spikes being counted during a stimulus interval (0.73 sec). Before each series of 360 stimulus presentations, the spontaneous discharge rate was sampled for ten 0.73 sec intervals. The average count was then subtracted from the counts recorded during each stimulus presentation. This procedure corrected for any shifts in the spontaneous firing rate during the experiment. The Mann-Whitney U-Test and Wilcoxon Matched-Pairs Signed-Rank Test (Siegel, 1956, pages 75 and 116) were used to assess the statistical significance of any response change. A reference to "statistical significance" indicates a probability error of 0.05 or less (two-tailed test).

RESULTS

Response decrements during acquisition

The initial responses to the first few air puffs were marked, but the number of spikes decayed rapidly during the first minute of stimulation. Most of the response decrement in all preparations took place during the first 60 puffs. This period is plotted in Figure 1 ("acquisition") for the three major groups. The

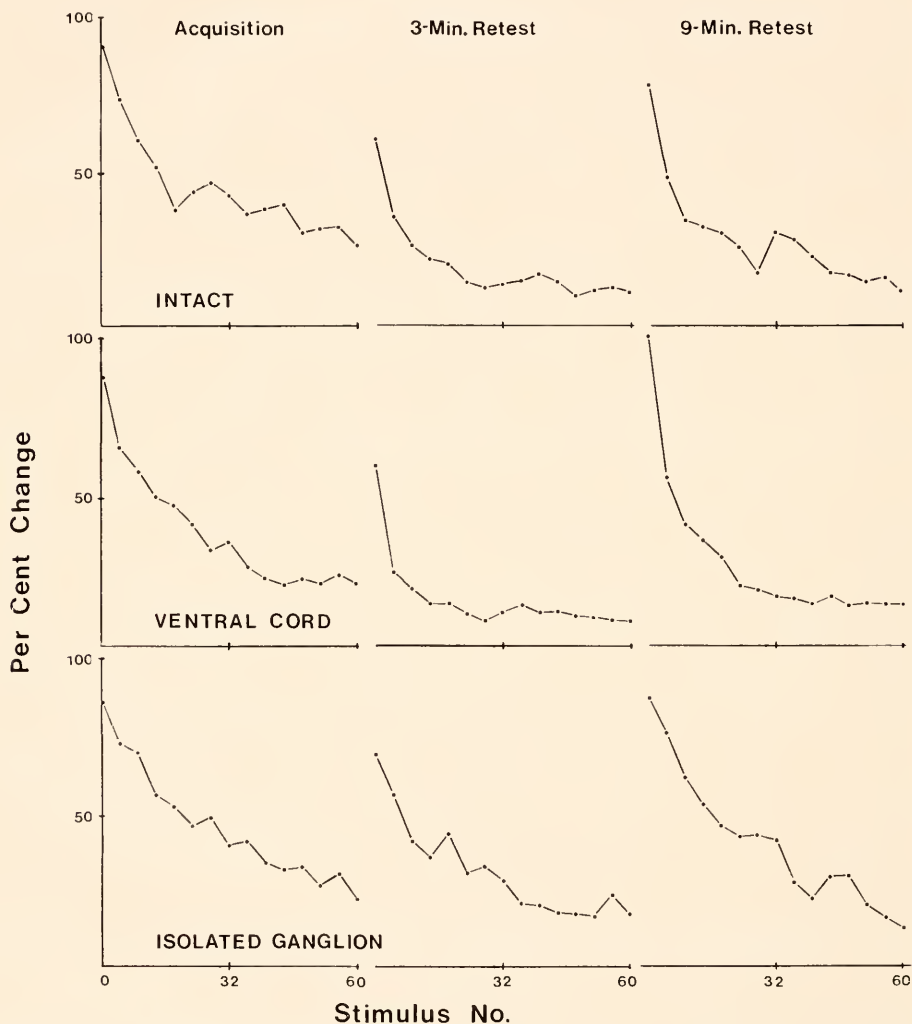


FIGURE 1. Median per cent changes in unit counts for the first 60 air puffs in all animals. The data are represented as per cent values; the initial (maximal) spike count recorded during the first air puff was used as the reference point (100%). All counts have been corrected with respect to any shifts in the baseline discharge rate recorded between series of stimulus presentations.

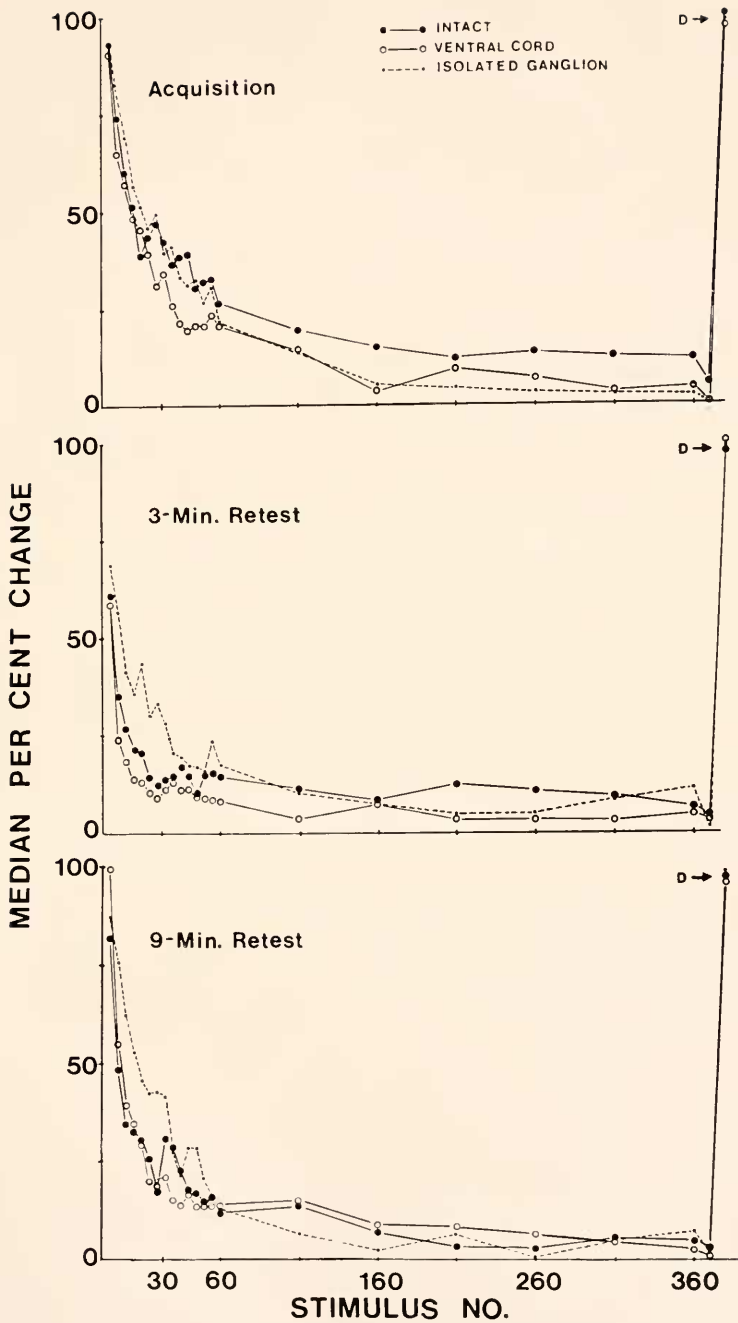


FIGURE 2. Comparisons of the Intact, Ventral Cord, and Isolated Ganglion groups during acquisition and retesting. "D" indicates the application of a different stimulus (usually a water drop) to the area that had been receiving the air puff.

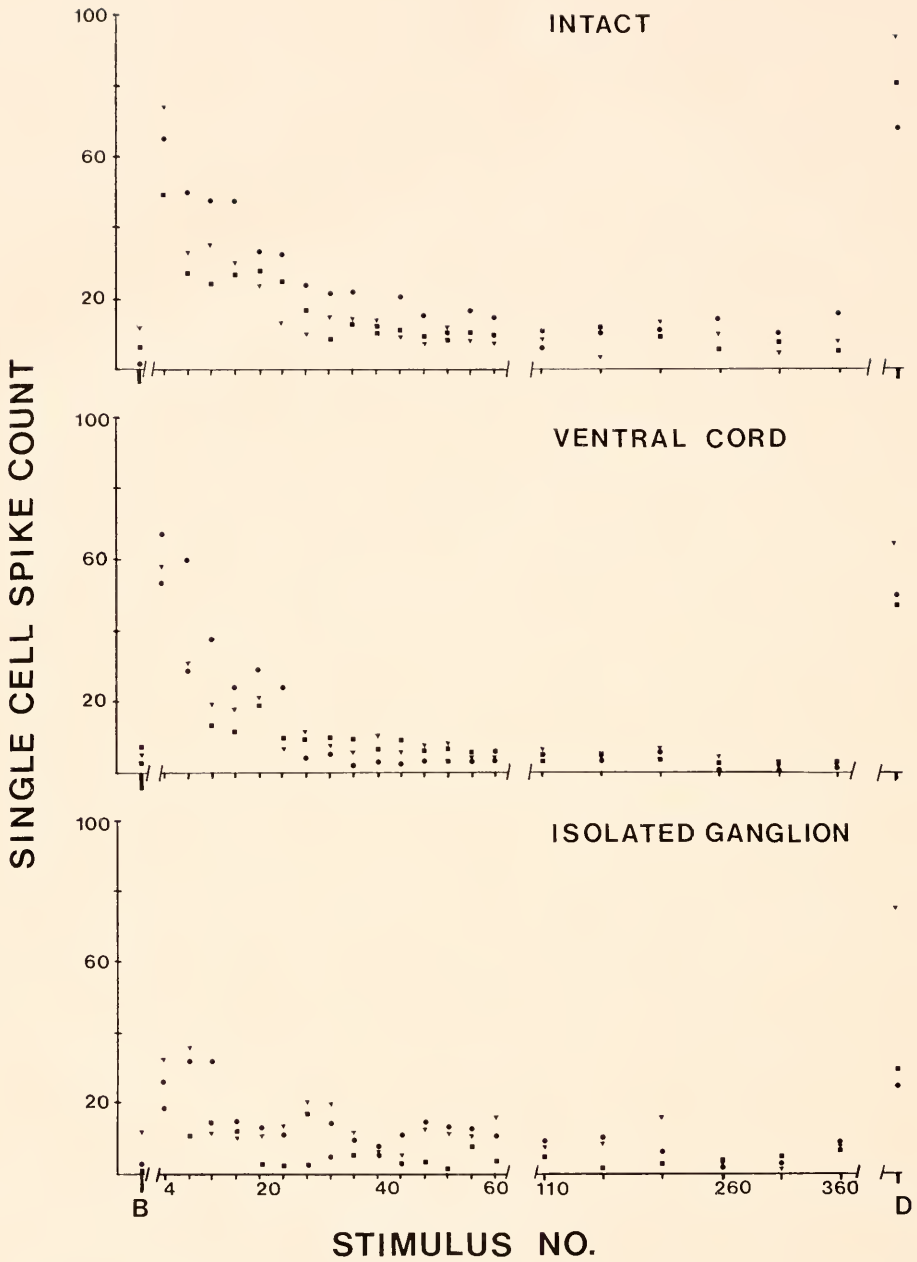


FIGURE 3. Examples of spike counts in single, identifiable dorsal nerve units in 3 different preparations (Intact, Ventral Cord and Isolated Ganglion) during acquisition and subsequent retests. These counts are uncorrected with respect to baseline changes. This correction amplifies differences between the acquisition and subsequent retest stages. The circle denotes acquisition; the square, 3-min retest; and the inverted triangle, 9-min retest.

data are represented as per cent values; the initial (and maximal) spike count recorded during the first air puff was used as the reference point (100%). It can be seen that in all three groups the repeated presentation of air puffs resulted in a decay of unit activity. Statistical tests indicate no significant difference between groups during the acquisition phase. A summary of the spike counts and the results of the statistical analyses for the first 60 trials are presented in Table I. After this initial and rapid response decrement, the counts in all groups leveled off and remained low for the remaining 300 stimulations (Fig. 2).

In Figure 3 we have included examples of uncorrected spike counts obtained in single, identifiable dorsal nerve units. Each of the 3 preparations is represented. These examples are preparations demonstrating moderate differences between the spike counts obtained during the initial acquisition phase and those obtained during the subsequent retest. The lower count observed in the isolated ganglion example of Figure 3 was typical in preparations of this group.

Measures of "retention"

After a 3-min respite the preparations in each group were presented another 360 stimulations to determine whether there was any persistence or retention of the response diminution over time. In all three groups there were a significantly fewer number of spikes during the first 60 trials of the 3-min retest when compared to the counts observed during the initial acquisition. In Figure 1, examination of the 3-min retest phase indicates that the initial responses of the groups were lower, and that the slopes were steeper. These lower response levels were statistically significant for the "Intact" group ($P < 0.01$), the "Ventral Cord" group ($P < 0.01$), and the "Isolated Ganglion" animals ($P < 0.05$). The individual subject totals in Table I show that for the Intact and Ventral Cord groups, 28 out of 29 subjects displayed a lower spike count during the 3-min retest. The persistence of a response decrement was not as great for the "Isolated Ganglion" preparations. Statistical analyses showed that both the Intact and the Ventral Cord groups were significantly less than the Isolated Ganglion group during the 3-min retest ($P < 0.05$ and $P < 0.02$, respectively). As in the acquisition phase, the response levels of these groups remained low for the remaining 300 stimulations.

A period of 9 minutes was allowed to elapse after completion of the 3-min retest before the third series of 360 air puffs was delivered. This 9-min retest provided information concerning a relatively long-term persistence of the response decrements and a general estimate of recovery time. In all groups there was an increase in the spike counts during the first 60 trials, but this was not significant (Fig. 1, Table I). Comparisons of the counts obtained during acquisition and those obtained during the first 60 trials of the 9-min retest yielded significantly lower scores for the Intact and Ventral Cord groups ($P < 0.02$; $P < 0.05$), but the Isolated Ganglion counts were returning to levels observed during acquisition.

"B" indicates the pre-stimulus spontaneous discharge rate; "D" indicates the application of a dishabituating stimulus. These examples are preparations showing moderate differences between the initial habituation and the retests.

TABLE 1
Data summary and statistical analysis for the first 60 puffs

Subject Numbers		Acquisition		3-minute retest		9-minute retest		Comparisons*	P
Intact	1	A	154	B	05	C	37	A-B	0.01
	2		108		57		66		
	3		89		60		114		
	4		65		21		55		
	5		65		28		17		
	6		72		09		16		
	7		47		07		16		
	8		48		04		37		
	9		18		02		15		
	10		103		74		54		
	11		101		46		16		
	12		90		88		37		
	13		293		240		102		
	14		84		39		93		
Total Spikes			1272		680		775		
Ventral cord	1	D	89	E	56	F	71	D-E	0.01
	2		45		16		90		
	3		216		86		55		
	4		327		126		99		
	5		289		01		00		
	6		59		15		20		
	7		08		10		07		
	8		84		49		38		
	9		95		39		27		
	10		229		93		231		
	11		39		14		57		
	12		65		53		65		
	13		90		18		87		
	14		33		33		73		
	15		290		23		60		
Total spikes			1958		632		980		
Isolated ganglion	1	G	18	H	72	I	25	G-H	0.05
	2		50		26		49		
	3		44		08		01		
	4		106		72		26		
	5		37		27		15		
	6		71		-19		06		
	7		37		46		27		
	8		42		19		28		
	9		86		75		99		
	10		100		35		98		
	11		75		106		117		
	12		69		38		57		
	13		278		73		133		
	14		11		06		12		
Total spikes			1024		584		693		
		Comparisons**	P	Comparisons**	P	Comparisons**	P		
		A-D	N.S.	B-E	N.S.	C-F	N.S.		
		A-G	N.S.	B-H	0.05	C-I	N.S.		
		D-G	N.S.	E-H	0.02	F-I	N.S.		

Comparisons of the three groups during the three phases of experimentation can be made from the data presented in Figure 2. In general, it appears that all groups were equal with respect to the initial acquisition of the habituation, but that the Isolated Ganglion group demonstrated less retention during the first

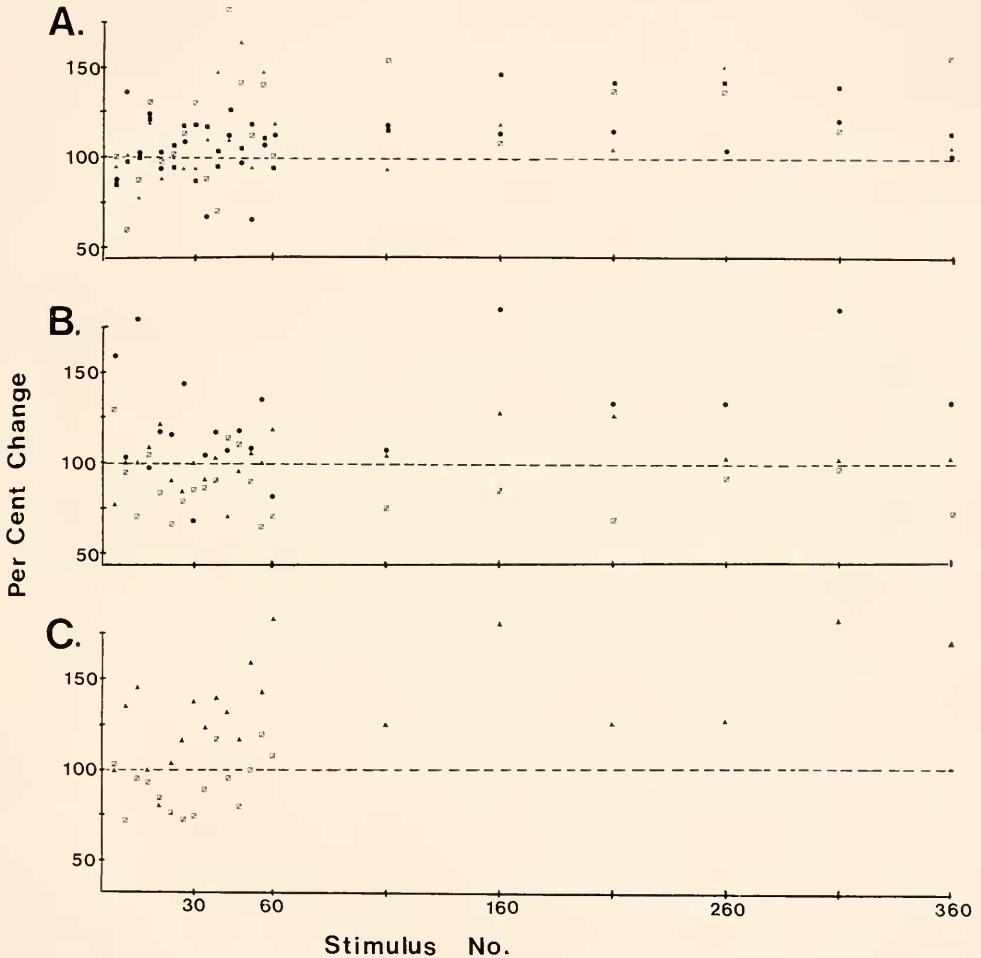


FIGURE 4. Examples of sensory nerve (ventral root) responses to air puffs. Each character (triangle, circle, etc.) represents the data obtained from a single subject. Each point is based upon an average count obtained over 4 stimulations. A, B, and C represent three blocks of 360 stimulations each, with rest periods of 3-min. and 9-min. between A-B and B-C, respectively. The initial response of the sensory nerve during the first air puff was used as the 100% reference point.

* Wilcoxon Matched-Pairs Signed-Ranks (Siegel, 1956).

** Mann-Whitney U-Test (Siegel, 1956).

All tests are two-tailed.

60 stimulations in the 3 and 9-min retention tests. There were no differences between preparations with respect to the number of neuromeres between the stimulated gill book and the recorded ganglion dorsal nerve output. Stimulation and recording at the same segment or across two or more segments led to similar response changes during acquisition and during the two retests. Accordingly, the data of these preparations were combined within the Intact and Ventral Cord groups.

Measures of "dishabituation"

A critical control for fatigue in demonstrations of habituation is to present a stimulus of different modality or intensity. A "dishabituation" or response recovery is typically obtained. In Figure 2 the results of such tests are represented. The application of water drops, tactile stimulation with a probe, or a more intense air puff in the spot that had been receiving repetitive air puffs, produced dorsal nerve responses that equalled the spike counts obtained when these nerves were first stimulated. This demonstration of response recovery rules against fatigue in dorsal nerve output or intra-ganglionic processes as an explanation for the response decrement.

Sensory nerve responses

Recordings made from fiber branches mediating tactile input show that the repetitive stimulation of the gill book surface with air puffs did not result in any drop in the spike counts elicited by each puff (Fig. 3). If anything, the spike counts obtained from four preparations show a slight increase over the 360 stimulations. Continuation of the stimulation in retests of some of these preparations failed to demonstrate any diminution in sensory nerve response.

DISCUSSION

Although habituation is observed in a wide variety of vertebrate and invertebrate species, certain general characteristics are common. Primarily, there is a rapid response decrement to a repetitively applied stimulus, a decrement that persists for a time, followed by a return to the original response tendencies or what is referred to as "spontaneous recovery." The post-habituation recovery emphasizes the relatively labile nature of this type of system adaptation. However, some portion of the habituation process does have a more stabile character. Frequently, a "potentiation of habituation" is observed where, after repeated habituation and spontaneous recovery cycles, the rate of habituation increases while the initial response levels may remain high. These various characteristics are usually distinguished from fatigue and sensory adaptation by two procedures. First, the application of a stimulus of a different type or intensity produces "dishabituation" or an immediate recovery of the response, thus ruling out fatigue. Secondly, analyses of afferent systems indicate that the response decrements are not due to a failure in sensory transmission. The above criteria, discussed by Thompson and Spencer (1966), have been met in the present investigations with *Limulus* abdominal ganglia. With repetitive tactile stimulation there is a rapid and persisting

drop in dorsal nerve unit activity; subsequent retests indicate a potentiation of habituation for at least 9 minutes. After habituation the nerve is still responsive to a different stimulus, indicating dishabituation; the sensory nerves do not show a decay in activation with repetitive tactile stimulation. Habituation then, is demonstrated at the neuronal level in what is considered to be a living fossil.

A similar demonstration of habituation in the abdominal ganglion of an arthropod was performed by Pumphery and Rawdon-Smith (1937) in the cockroach. Repetitive stimulation of the cercus at particular frequencies resulted in what was termed "adaptation" of the ganglionic response recorded in the post-synaptic nerves, while the preganglionic afferent responses remained constant. The interpolation of one extra pulse in the stimulus train resulted in dishabituation—the post-synaptic response returned to initial levels. This degree of stimulus-specificity is unusual. Additional electrophysiological evidence for habituation in the isolated abdominal ganglion of the cockroach is reported by Hughes (1965). Stimulation of the annal cerci with air puffs at 1/sec resulted in a diminution of the giant fiber discharge with the major decay in responsivity occurring during the first 15 secs of stimulation. Recordings from the cercal nerve showed a constant rate of sensory input. A series of retests indicated some retention over time. Evidence for long-term retention of habituation in arthropods was obtained by Rowell (1968), who studied response decrements of units in the tritocerebrum of locusts. Successive presentation of stimuli (a moving black disc) was followed by a drop in unit discharge. After a 5-min interval, the stimulus series was presented again, and a more rapid decay in unit activity took place. After 4 such series the animal was allowed a 2-hour rest, and in the subsequent series the units still demonstrated an initially lower response level and more rapid habituation. In *Limulus* abdominal ganglia, a 9-min retention span was observed in the intact system and intact ventral cord region. The isolated ganglion showed little carry-over of the previous habituation in the second (9-min) retest, although there was significant retention in the 3-min retest.

Comparisons between the major groups suggest no relationship between neural complexity and initial acquisition. All groups demonstrated similar decays in unit activity during the first 360 presentations. There is, however, some relationship between neural complexity and the persistence or retention of the response decrement in the retests. While the intact and isolated cord groups displayed a persistence effect on the 9-min retest, the isolated ganglion group had almost returned to levels observed in the initial acquisition series. The response decay during the 9-min retest was similar to that recorded in the initial series of stimulus presentation. This loss of the response decrement may well be due to a metabolic deterioration. In our previous work on cardioregulatory properties of the abdominal ganglia (Corning and Von Burg, 1968; Von Burg and Corning, 1970), we had frequently noted that the experimental "lifetime" of a ganglion was shortened when it was isolated from the system. This possible deterioration does not affect acquisition, since habituation still occurred during the 9-min retest in the Isolated Ganglion group. It may well be, as Applewhite and Gardner (1971) have suggested, that acquisition of habituation is metabolically independent. It was found, for example, that the rate of habituation was independent of temperature and unaffected by inhibition of protein and RNA synthesis, while

response recovery was apparently metabolically dependent. However, the work of Applewhite was on a protozoan, and the mechanisms involved during the acquisition phase may not be the same for the arthropod. Such hypotheses concerning the physiological and biochemical mechanisms underlying habituation could be tested in the abdominal ganglion preparation of *Limulus*. The arterial sheath surrounding the cord permits relatively easy and localized perfusion (Corning and Von Burg, 1970), and the persistence of a response decrement for 9-min is sufficient time for the temperature of the system to be altered or for the perfusion of agents that might disrupt or enhance the persistence of a response decrement.

SUMMARY

1. Extracellular recordings were made from dorsal root units in the abdominal ganglia of *Limulus*. These units are activated by tactile stimulation of the gill book covers.

2. The response in these units to repeated puffs of air directed on a gill book is a rapid decline in activity. The response "spontaneously recovers" with time when the stimulus is withheld. Stronger stimuli or different stimuli (water drops) produce an immediate recovery of the diminished responsivity; this recovery is similar to "dishabituation" noted in other studies and rules out fatigue as an explanation for the dorsal root response decrement. Sensory fatigue or adaptation are ruled out by the demonstration that repeated puffs of air do not produce any response decrement in the nerves (ventral roots) which carry the afferent tactile information.

3. The relationship between the number of ganglia and the acquisition of habituation was examined and no differences were found. However, during retests the Intact and Isolated Ventral Cord preparations demonstrated a better persistence of the response decrement.

4. These findings are discussed with respect to habituation data obtained by others in invertebrates.

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